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Sildenafil ve Bacillus Clausii'nin Metotreksat Bağlı Mukozitin Önlenmesindeki Rolü: Preklinik Çalışma

The role of Sildenafil and Bacillus clausii for the Prevention of Methotrexate Induced Mucositis: A Preclinical Study

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ÖZ

Amaç: Metotreksat (MTX), klinik onkoloji pratiğinde ve otoimmün hastalıkların tedavisinde yaygın olarak kullanılan bir kemoterapötik ajanıdır. Tedavi sırasındaki ana yan etkisi mukozittir. Son zamanlarda yayınlanan çalışmalar, bu etkinin doğrudan bağırsak epiteli hasarına ve bağırsak mikrobiyotasının bozulmasına bağlı olabileceğini bildirmiştir. Bu çalışmada, MTX'ın neden olduğu bağırsak histolojik değişikliklerini karakterize etmeyi ve bir sıçan modelinde Sildenafil' in ve probiyotik *Bacillus clausii'* nin bağırsak mukozit üzerindeki etkilerini ve güvenliğini araştırmayı hedefledik.

Materyal ve Metot: Sıçanlar 4 gruba ayrıldı; Grup 1 (n=8) MTX + *B. clausii* aldı, Grup 2 (n=8) sadece MTX aldı, Grup 3 (n=8) MTX + Sildenafil aldı ve Grup 4 (n=7) kontrol olarak kullanıldı (tedavi almadı).

Bulgular: MTX + *B.clausii* grubunda villüs atrofisi, villöz füzyon ve erozyon oranı, MTX grubu ve MTX + Sildenafil grubuna göre istatistiksel olarak anlamlı derecede düşük bulundu.

Sonuç: Çalışma, MTX tedavisinden sonra *B. clausii* 'nin bağırsak mukozasının onarımı üzerindeki etkisini gösterdi. Probiyotiklerin intestinal mukozal koruma üzerindeki etkilerinin daha iyi anlaşılması, kemoterapiye bağlı mukozitte yeni terapötik yaklaşımlara yol açabilir.

Anahtar Kelimeler: Bacillus clausii, metotreksat, probiyotik, sıçan, sildenafil

ABSTRACT

Objective: Methotrexate (MTX) is a commonly used chemotherapeutic agent in clinical oncology practice and in treatment of autoimmune diseases. The major side effect during treatment is mucositis. Recently published studies reported that this effect may be due to direct intestinal epithelial injury and disturbance of intestinal microbiota. In this study, we aimed to characterize the intestinal histological changes caused by MTX and to investigate the effects and safety of Sildenafil and probiotic *Bacillus clausii* on intestinal mucositis in a rat model.

Materials and Methods: The rats were divided in 4 groups; Group1(n=8) received MTX + *B. clausii*, Group 2 (n=8) received only MTX, Group3 (n=8) received MTX + Sildenafil and Group 4 (n=7) was served as control (received no treatment).

Results: The rate of villus atrophy, villous fusion and erosion in the MTX + *B. clausii* group were found to be statistically significantly lower than in the MTX group and the MTX + Sildenafil group.

Conclusion: The study demonstrated the effect of *B. clausii* on repairing of intestinal mucosa after MTX-treatment. The better understanding of the effects of probiotics on intestinal mucosal protection may lead to new therapeutic approaches in chemotherapy-induced mucositis.

Keywords: Bacillus clausii, methotrexate, probiotic, rat, sildenafil

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INTRODUCTION

Methotrexate (MTX) is a chemotherapeutic agent and an immunosuppressant. It is the first-line therapy used in the treatment of autoimmune and oncologic disease in children. MTX also is an antibacterial drug metabolized by human gut bacteria. MTX administration is associated with multiple adverse drug reactions (ADRs), such as gastrointestinal toxicity. Intestinal mucosal damage is the most common side effect of chemotherapeutics. A recently published study reported the mucosal injury in MTX -treated mice, leading to significant changes in macrophages and also disturbances in gut microbiota. *Bacteroides fragilis* was significantly decreased after MTX treatment. The authors showed a strong impact of the gut microbiota on MTX-induced intestinal mucositis.[1,2] Gut microbial dysbiosis is an alteration of composition and functions of intestinal microbiota, which contributes to the onset of many disorders like inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), type 1 diabetes, obesity, atopy and allergies.[3] Drugs (i.e. antibiotics, anticancer drugs) may cause dysbiosis.[4,5] Recently published studies reported that probiotics are recognized to be effective and safe in restoring gut microbiota dysbiosis. Probiotics have been shown to have many beneficial effects: by its immunomodulatory effects can regulate the mucosal permeability and prevent drug-induced mucosal damage and bacterial translocation. Probiotics have the capacity to modulate the mucosal low-grade inflammation and eventually the consequences of the "leaky gut".[4-8]

Sildenafil is a strong and selective inhibitor of cGMP-specific phosphodiesterase type 5 (PDE5). It has been shown that Sildenafil have positive effects on intestinal adaptation parameters, especially in the jejunum, in the short bowel syndrome in rats, and increase the villi length and cryptic depth in the intestine. These effects have explained by increasing nitric oxide.[9,10]

In this study, we aim to characterize the intestinal histological changes caused by MTX and to investigate the efficacy and safety of probiotics and Sildenafil on intestinal mucositis in a rat model.

MATERIALS AND METHODS

The study was conducted in the Experimental Laboratory at Bezmialem Vakıf University. All procedures were performed according to the Guide for the Care and Use of Laboratory Animals. All protocols were approved by the Institutional Animal Care and Use Committee of the University (Date:22.02.17, decision no:15) and followed the institutional guidelines for the care and use of laboratory animals.

For the study, 31 healthy male Wistar Albino rats weighing 250 ± 20g were used. Rats were kept under standard laboratory conditions in rooms with a constant temperature of 23 ±2 ° C and a humidity of 60 ± 5%, exposed to sunlight and ventilated. Standard feed and water were released during the study period. The rats were divided in 4 groups; Group1 (n=8) received MTX + *B. clausii*, Group 2 (n=8) received only MTX, Group3 (n=8) received MTX + Sildenafil and Group 4 (n=7) was served as control (received no treatment). Only the oral standard feed and water was given to the control group. The single dose of MTX (20 mg/kg) was injected intraperitoneally (i.p.). Sildenafil (Viagra, Pfizer) was dissolved in water and administered at a dose of 60 mg/kg for 3 days and 1 vial of *B. clausii* (2X109 CFU, Sanofi, Enterogermina) for 3 days via oral gavage, starting one day after MTX injection. All rats were euthanized by giving overdose sodium pentothal (200mg/kg, intramuscular) at the end of the 3rd day and sacrificed, then midline laparotomy was performed. For histological examination, a 5 cm small intestinal and ascending colon specimens were taken 10 cm proximal and 10 cm distal from the ileocecal valve. Luminal contents were washed with 0.9% NaCl by injecting saline through the lumen of the ileum and colon segments. Tissue samples were fixed in 10% buffered formalin, dehydrated with alcohol, and embedded in paraffin, then cut in 5µm sections and stained with hematoxylin eosin (HE) and Periodic Acid-Schiff-Alcian Blue (pH:2.5) (PAS). The specimens were assessed under a light microscope by the same experienced pathologist blinded to the groups. Microscopically, cryptitis, crypt abscess, apoptosis, loss of goblet cell, villous atrophy, increased in the infiltration of lymphocyte and neutrophils in lamina propria were graded as 0: none, 1: mild, 2: moderate, 3: severe. Epitheliitis, erosion, ulceration, crypt distortion, basal plasmacytosis, granuloma, increased in lymphoid follicles/ aggregate were graded as none/yes. Villous fusion was graded none/yes on the small intestine specimen; villous formation was graded none/yes on the large intestine specimen. All photomicrographs were taken under the light microscope with image analysis software equipment attached (Olympus Application Software DP-2BSW)

Statistical Analyses: IBM SPSS Statistics 22 (IBM SPSS, Turkey) program was used for statistical analysis while evaluating the findings obtained in the

study. The study data was used in comparing identifying statistical methods (frequency). In addition to comparing qualitative evaluation, the Chi Square Test, the Fisher's Exact test and the Fisher Freeman Halton test were used. Significates were evaluated at $p<0.05$.

RESULTS

All rat tolerated the experiments well, and no adverse effects observed. The small intestine and colon biopsies of 31 rat were evaluated. Pathological changes caused by MTX were evaluated in the small intestine (from pyloric sphincter to ileocecal sphincter) and colon (from ascending colon to rectum).

In the Small Intestine: There was not statistically significant difference between the groups in terms of distribution rates of cryptitis, crypt abscess, apoptosis, goblet cell and increase in lymphocytes ($p>0.05$). Among the groups, there was a statistically significant difference between the groups in terms of distribution of villus atrophy rates ($p:0.011$; $p<0.05$). As a result of the bi-level comparisons made for the detection of difference; the rate of villus atrophy in the MTX + *B. clausii* group were found to be statistically significantly lower than in the MTX group and the MTX + Sildenafil group ($p:0.007$; $p<0.05$). The rate of villus atrophy in the control group, were found to be statistically significantly lower than in the MTX group and in the MTX + Sildenafil group ($p:0.013$; $p<0.05$). Among other groups, there was no statistically significant difference in terms of distribution rates of villus

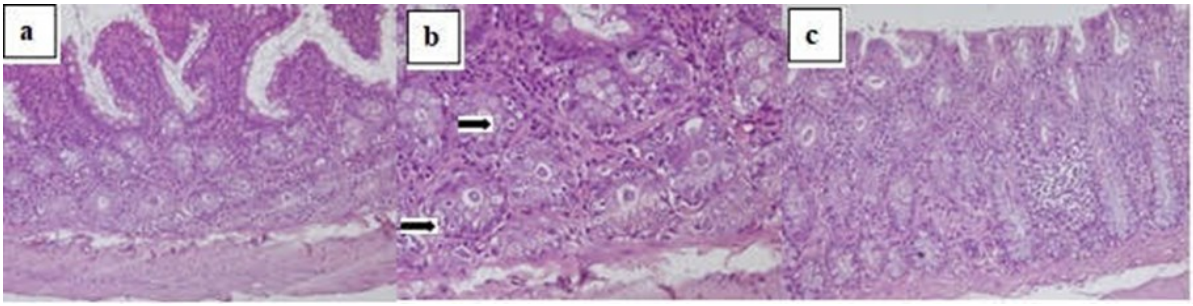

Figure 1. Histological examination findings in MTX + *B. clausii* group.

Small intestine: a. Mild villous atrophy, lymphocytes, and plasma cells infiltration and apoptosis (H&Ex10); b. Loss of Paneth cell and apoptosis (arrow) in crypt epithelium (H&Ex20);Colon: c. lymphoid aggregates and sparse neutrophil infiltration(H&Ex10).

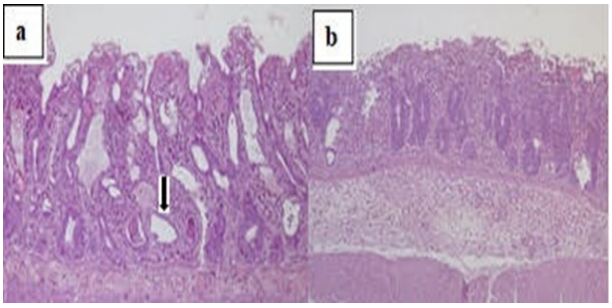

Figure 2. Histological examination findings in MTX group.

Small intestine: a. Moderate villous atrophy and fusion, crypt abscess, ghost crypts (arrow), infiltration of lymphocytes and neutrophils in lamina propria (H&Ex20); Colon: b. Erosion, crypt abscess, infiltration of lymphocytes and neutrophils in mucosa and submucosa (H&Ex10).

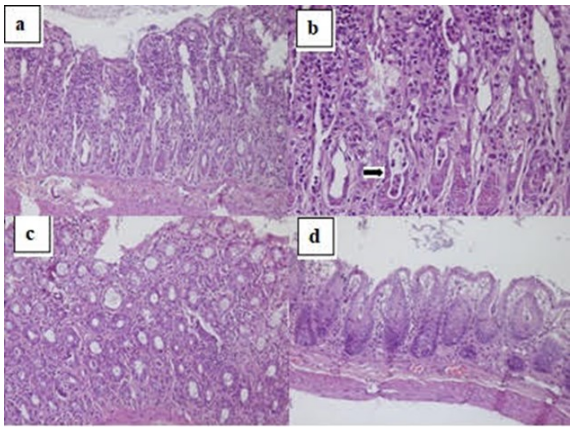

Figure 3. Histological examination findings in MTX-Sildenafil group.

Small intestine: a. Moderate villous atrophy, crypt abscess, lymphocytes, and eosinophils in lamina propria (H&Ex10); b. Shrinkage of Paneth cells, and apoptotic cells and neutrophils into the crypt lumens (arrow) (H&Ex20); Colon: c. Degenerative changes of crypts, and lymphocytes and neutrophils in lamina propria (H&Ex10); d. Loss of goblet cells on the surface

Table 1. Histological evaluation of parameters between groups in the small intestine.

Small intestine	Parameters	Group 1 MTX + *B.clausii* n (%)	Group 2 MTX n (%)	Group 3 MTX + Sildenafil n %()	Group4 Control n (%)	p[a]
Cryptitis	**None**	5 (62.5)	3 (37.5)	3 (37.5)	7 (100)	0.286
	Mild	2 (25)	1 (12.5)	1 (12.5)	0 (0)	
	Moderate	1 (12.5)	2 (25)	2 (25)	0 (0)	
	Severe	0 (0)	2 (25)	2 (25)	0 (0)	
Crypt abscess	**None**	7 (87.5)	3 (37.5)	2 (25)	7 (100)	0.080
	Mild	0 (0)	1 (12.5)	2 (25)	0 (0)	
	Moderate	1 (12.5)	2 (25)	1 (12.5)	0 (0)	
	Severe	0 (0)	2 (25)	3 (37.5)	0 (0)	
Apoptosis	**None**	6 (75)	4 (50)	3 (37.5)	7 (100)	0.428
	Mild	1 (12.5)	1 (12.5)	1 (12.5)	0 (0)	
	Moderate	1 (12.5)	1 (12.5)	2 (25)	0 (0)	
	Severe	0 (0)	2 (25)	2 (25)	0 (0)	
Villus atrophy	**None**	8 (100)	2 (25)	2 (25)	7 (100)	**0.011***
	Mild	0 (0)	3 (37.5)	2 (25)	0 (0)	
	Moderate	0 (0)	2 (25)	1 (12.5)	0 (0)	
	Severe	0 (0)	1 (12.5)	3 (37.5)	0 (0)	
Increase in lympho-cytes	**None**	4 (50)	3 (37.5)	2 (25)	7 (100)	0.073
	Mild	4 (50)	4 (50)	4 (50)	0 (0)	
	Moderate	0 (0)	1 (12.5)	2 (25)	0 (0)	
Increase in neutro-phils	**None**	8 (100)	4 (50)	4 (50)	7 (100)	**0.022***
	Mild	0 (0)	1 (12.5)	4 (50)	0 (0)	
	Moderate	0 (0)	1 (12.5)	0 (0)	0 (0)	
	Severe	0 (0)	2 (25)	0 (0)	0 (0)	
Goblet cell loss	**None**	8 (100)	5 (62.5)	3 (37.5)	7 (100)	0.173
	Mild	0 (0)	1 (12.5)	1 (12.5)	0 (0)	
	Moderate	0 (0)	0 (0)	1 (12.5)	0 (0)	
	Severe	0 (0)	2 (25)	3 (37.5)	0 (0)	

[a]: Chi square Test; *: $p<0.05$.

atrophy ($p>0.05$) (Figre1 a, b, Figure 2 a, b, Figure 3 a, b).

There was a statistically significant difference between the groups in terms of distribution rates of increase in neutrophils (p:0.022; $p<0.05$). As a result of bi-level comparisons for the determination of diversity; the mild increase in neutrophils in the MTX + *B. clausii* group was found to be statistically significantly lower than in the MTX + Sildenafil group (p:0.038; $p<0.05$). Among other groups, there is no statistically significant difference in terms of increase in neutrophils distribution rates ($p>0.05$) **(**Table 1**)**.

In the Colon: There was not statistically significant difference between the groups in terms of distribution rates of cryptitis, crypt abscess, apoptosis, increase in neutrophils and Goblet cell loss ($p>0.05$).

Among the groups there was a statistically significant difference in terms of distribution rates of increase in lymphocytes levels (p:0.027; $p<0.05$). As a result of bi-level comparisons for the determination of differences; the rate of mild increase in lymphocytes in the MTX + Sildenafil group (62.5%), were found to be statistically significantly higher than in the MTX group (0%) and the Control group (0%) (p:0.026; $p<0.05$) (Figure1c, Figure 3c, d).

In the Small Intestine: There was not statistically significant difference between the groups in terms of the incidence of epitheliitis, crypt distortion and increase in lymphoid follicles/ aggregate incidence ($p>0.05$). There was a statistically significant difference between the groups in terms of erosion incidence (p:0.034; $p<0.05$). As a result of bi-level comparisons for the determination of difference; the rate of erosion in the MTX +*B. clausii* group, was found to be statistically significantly lower than in the MTX + Sildenafil group (p:0.038; $p<0.05$). There was not statistically significant difference between other groups in terms of erosion incidence ($p>0.05$).

There was a statistically significant difference between the groups in terms of the incidence of vil-

Table 2. Evaluation of biopsy parameters between groups in the small intestine and colon.

	Parameters	Group 1 MTX + *B.clausii*	Group 2 MTX	Group 3 MTX + Sildenafil	Group4 Control	p[a]
		n (%)	n (%)	n (%)	n (%)	
Small Intestine	Epitheliitis	0 (0)	1 (12.5)	0 (0)	0 (0)	1.000
	Erosion	0 (0)	1 (12.5)	4 (50)	0 (0)	**0.034***
	Villous fusion	0 (0)	4 (50)	1 (12.5)	0 (0)	**0.034***
	Crypt distortion	0 (0)	1 (12.5)	2 (25)	0 (0)	0.587
	Increase in Lymphoid follicles/ aggregate	2 (25)	2 (25)	2 (25)	0 (0)	0.566
	Erosion	0 (0)	1 (12.5)	0 (0)	0 (0)	1.000
	Villous formation	0 (0)	0 (0)	4 (50)	0 (0)	**0.008***
	Crypt distortion	0 (0)	1 (12.5)	0 (0)	0 (0)	1.000
	Increase in Lymphoid follicles/ aggregate	3 (37.5)	0 (0)	1 (12.5)	0 (0)	0.157

[a]:Fisher Freeman Halton Test; *p<0.05.

lous fusion (p:0.034; p<0.05). The rate of villous fusion in the MTX +*B. clausii* group was found to be statistically significantly lower than in the MTX group (p:0.038; p<0.05). Among other groups, there is no statistically significant difference in the rates of villous fusion (p>0.05).

In the Colon: There was not statistically significant difference between the groups in terms of erosion, rates of crypt distortion and increase in lymphoid follicles/ aggregate incidence (p>0.05). There was a statistically significant difference between the groups in terms of the incidence of villous formation (p:0.008; p<0.05). The rate of villous formation in the MTX + Sildenafil group was found to be statistically significantly higher than in the MTX +*B. clausii* and MTX group (p:0.038; p<0.05). Among other groups, there was not statistically significant difference in the rates of villous formation (p>0.05) (Table 2).

DISCUSSION AND CONCLUSION

MTX, an inhibitor of dihydrofolate reductase and DNA synthesis, is a commonly used chemotherapeutic agent in clinical oncology practice and in treatment of autoimmune diseases. Two major side effects during treatment are mucositis and diarrhea. This side effects directly influencing the success of therapy and patient's compliance to treatment. The mechanisms by which MTX causes intestinal damage are not fully understood. MTX can induce intestinal mucositis by direct injury to intestinal mucosa and cause significant oxidative stress.[11] Treatment with MTX induced villous atrophy and fusion, crypt loss, neutrophil infiltration in the lamina propria and goblet cell depletion.[12] The intestinal mucus layer plays an important role in intestinal barrier function. Decreased goblet cells reducing mucin secretion and disturbing the protective effect of the mucosal barrier. In this study, although not statistically significant, goblet cell loss in MTX group was 62.5%, in MTX + Sildenafil group was 37.5% and no loss was observed in MTX + *B. clausii* group.

Also MTX administration lead to alterations in the composition, diversity, and functions of the intestinal microbiota, especially *Bacteroides.*[13] The alterations in the composition of the gut microbiota (dysbiosis) caused by chemotherapy treatment plays role in the pathophysiology of mucositis. The restoration of intestinal microbiota by probiotics could ameliorate inflammation and reduce the severity of chemotherapy-induced mucositis.[14-16] Recently published study had demonstrated the therapeutic effects of probiotics on modulation of abundance and diversity of gut microbiota on 5-Fluorouracil (5-FU) induced mucositis in a mouse model.[17] The immunomodulatory effect of *B. clausii* could be the result of the inducing NOS II synthetase activity, IFN-gamma production, and CD4+ T-cell proliferation.[18] Treatment with *B. clausii* can attenuated histopathological changes of mucosal injury induced by 5-FU in mice, improved the decrease in villus/crypt ratio, decreased neutrophil infiltration and recovered altered gastrointestinal motility.[19]

In our study, the MTX-treated groups showed a villous atrophy, erosions, increased number of lymphocytes in the colonic lamina propria. We demonstrate that treatment with probiotic *B. clausii* significantly reduced MTX-induced histopathological small intestinal changes; the rate of villus atrophy, the rate of erosion and villus fusion in the MTX + *B. clausii*

group were found to be statistically significantly lower than in the MTX group. Sildenafil failed to alleviate the villus changes in small intestine. No significant difference was observed between the groups in terms of cryptitis and crypt abscess. A recently published systematic review summarized the results of 15 human studies on the effect of probiotics on mucositis during chemo/radiotherapy. They found that probiotics can decrease the incidence rate of mucositis in cancer patients. The authors suggested that a combination of *Bifidobacterium longum*, *Lactobacillus acidophilus*, *Bifidobacterium breve*, *Bifidobacterium infantis*, and *Saccharomyces boulardii* could be a good combination for treatment of mucositis.[20]

There are several limitations in our study. We evaluated only the intestinal histological changes and did not investigate the influence of probiotics on microbiota composition and inflammatory/ proinflammatory cytokines. In the next phase of our study, we will also evaluate microbiota, metabolomics and cytokine changes.

In conclusion: In this study, we demonstrated the effect of *B. clausii* on repairing of intestinal mucosa after MTX-treatment. The better understanding of the effects of probiotics on intestinal mucosal protection may lead to new therapeutic approaches in chemotherapy-induced mucositis. More clinical trials are needed to define which probiotics or combinations are the best to reduce the rates of drug-induced mucositis.

Ethics Committee Approval: Our study was approved by the Institutional Animal Care and Use Committee of the Bezmialem Vakıf University (Date:22.02.17. decision no:15).

Conflict of Interest: No conflict of interest was declared by the authors.

Author Contributions: Concept – AK. SG; Supervision – AK. SG; Materials – CS. MA; Data Collection and/or Processing – EZ. SLM; Analysis and/ or Interpretation – EZ. AK. SG; Writing –AK. SG. EZ.

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